Biogeosciences

Influence of CO_2 and nitrogen limitation on the coccolith volume of *Emiliania huxleyi* (Haptophyta)

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Abstract. Coccolithophores, a key phytoplankton group, are one of the most studied organisms regarding their physiological response to ocean acidification/carbonation. The biogenic production of calcareous coccoliths has made coccolithophores a promising group for paleoceanographic research aiming to reconstruct past environmental conditions. Recently, geochemical and morphological analyses of fossil coccoliths have gained increased interest in regard to changes in seawater carbonate chemistry. The cosmopolitan coccolithophore *Emiliania huxleyi* (Lohm.) Hay and Mohler was cultured over a range of pCO_2 levels in controlled laboratory experiments under nutrient replete and nitrogen limited conditions. Measurements of photosynthesis and calcification revealed, as previously published, an increase in particulate organic carbon production and a moderate decrease in calcification from ambient to elevated pCO_2. The enhancement in particulate organic carbon production was accompanied by an increase in cell diameter. Changes in coccolith volume were best correlated with the coccosphere/cell diameter and no significant correlation was found between the coccolith volume and the particulate inorganic carbon production. The conducted experiments revealed that the coccolith volume of *E. huxleyi* is variable with aquatic CO_2 concentration but its sensitivity is rather small in comparison with its sensitivity to nitrogen limitation. Comparing coccolith morphological and geometrical parameters like volume, mass and size to physiological parameters under controlled laboratory conditions is an important step to understand variations in fossil coccolith geometry.

1 Introduction

Coccolithophores, a key functional phytoplankton group, evolved about 225 million years ago and their intracellularly produced coccoliths have been present in the sediment record ever since. Over geological times coccolithophores experienced various environmental conditions and are facing nowadays an alteration of the seawater carbonate chemistry due to the anthropogenic release of carbon dioxide. Atmospheric CO_2 is absorbed by the ocean and alters its carbonate chemistry towards an increase in dissolved inorganic carbon and a decrease in pH, referred to as ocean carbonation/acidification. The response of coccolithophores to elevated pCO_2 under nutrient replete conditions has been intensively studied in numerous controlled laboratory studies (Riebesell et al., 2000; Langer et al., 2006; Feng et al., 2008; Langer et al., 2009; Barcelos e Ramos et al., 2010; Krug et al., 2011). Especially, the cosmopolitan species *Emiliania huxleyi* is one of the most studied planktonic species in regard to ocean carbonation/acidification. Diverging results on *E. huxleyi* have triggered scientific discussions and a deeper reflection of the conducted experiments (Riebesell et al., 2008; Iglesias-Rodriguez et al., 2008; Shi et al., 2009).

However, recent results confirm a rather uniform response of *E. huxleyi* to $p\text{CO}_2$ under nutrient replete conditions with strain specific sensitivities (Langer et al., 2009; Bach et al., 2011; Findlay et al., 2011; Hoppe et al., 2011).

Studies on *E. huxleyi* under nutrient limited conditions and elevated $p\text{CO}_2$ are rare (Sciandra et al., 2003; Leonardos and Geider, 2005; Borchard et al., 2011), whereas light, macro- and micronutrient supply in the upper ocean are the main factors limiting phytoplankton growth (Davey et al., 2008; Moore et al., 2008; Marinov et al., 2010). *Emiliania huxleyi* is a poor competitor for nitrate compared to diatoms (Riegmann et al., 1992) but has an extraordinarily high affinity for orthophosphate and is able to utilise organic phosphates (Riegmann et al., 2000), displaying a high competitive ability in phosphate limited areas of the ocean. Nitrogen and phosphorus limitation lead to reduced growth rates and changes in cell diameter of *E. huxleyi*. Phosphate limited conditions cause an increase in cell diameter, whereas under nitrogen limitation the cell diameter decreases (Paasche, 2002; Müller et al., 2008). Inducing high $p\text{CO}_2$ levels on nitrogen limited cultures of *E. huxleyi* results in a further decrease in cell diameter (Sciandra et al., 2003).

The intracellularly produced coccoliths vary in volume and mass with coccolithophore species and strain (Young and Ziveri, 2000). Volume and weight estimates of coccoliths are used in paleoceanographic studies to estimate carbonate fluxes from the surface to the deep ocean (Young and Ziveri, 2000; Beaufort et al., 2007). Recently, changes in seawater carbonate chemistry over the last 40 000 years have been linked to the distribution of differentially calcified species and morphotypes (Beaufort et al., 2011) but the complexity of environmental factors triggering changes in coccolith geometry (mass, volume and size) is noted (Beaufort et al., 2011; Herrmann et al., 2012; Poulton et al., 2011).

Besides the complex interaction of environmental factors (temperature, nutrient availability, carbonate system, grazing pressure etc.) which have an influence on the species/strain composition of coccolithophore populations and additionally on the individual physiological control of coccolith geometry, indications are given for a linkage between the coccolith size (distal shield length) and the coccosphere diameter from fossil and field samples (Henderiks, 2008; Henderiks et al., 2012).

In this study, we used the cosmopolitan coccolithophore species *Emiliania huxleyi* to investigate the effect of changes in the seawater carbonate chemistry and nitrogen availability on the coccolith volume under controlled laboratory conditions.

2 Methods

2.1 Cultures

Emiliania huxleyi (Lohm.) Hay and Mohler (morphotype A) was isolated in 2009 in the Raune Fjord (Norway) by K. Lohbeck and cultured in natural seawater under nutrient replete conditions at $20\,°\text{C}$ and a light intensity of $300\,\mu\text{mol photons m}^{-2}\,\text{s}^{-1}$. The culture was kept under continuous light to desynchronise the cell division cycle. Desynchronization was checked by cell diameter measurements over 24 h via a Beckman Coulter Multisizer™3 (see below), whereby no significant change in cell diameter of the population was detected (data not shown). Culture media were prepared by filtrating (0.2 μm pore size) and subsequent autoclaving of Mediterranean sea water (salinity of 38). After autoclaving, seawater was bubbled with ambient sterile air (0.1 μm pore size) to reintroduce inorganic carbon and to equilibrate the carbonate system to ambient $p\text{CO}_2$ conditions. Precultures of *Emiliania huxleyi* were maintained under dilute batch culture conditions ($< 1.5 \times 10^5$ cells ml^{-1}) in exponential growth at ambient $p\text{CO}_2$ conditions with macro- and micronutrient addition corresponding to f/20 after Guillard (1975). Nitrate and phosphate concentrations were 88.2 and 3.6 μmol l^{-1}, respectively. Precultures of *E. huxleyi* were not acclimated to the applied $p\text{CO}_2$ conditions prior to the experiments (see below). However, studies indicate that *E. huxleyi* when exposed to new $p\text{CO}_2$ conditions displays after 8 h a similar physiological response compared to cultures acclimated for 10 or more generations (Barcelos e Ramos et al., 2010; Riebesell et al., 2000; Müller et al., 2010). It is therefore assumed that over the course of the conducted experiments (exponential growth of 5 or more generations) cells of *E. huxleyi* were fully acclimated to the experimental $p\text{CO}_2$ conditions at the time of sampling. As an important note of caution, it should be mentioned that precultures of *E. huxleyi* should be cultured in dilute exponential growth to avoid an elongation of the acclimation period due to a change of the physiological state caused by nutrient limitation or major changes in the carbonate chemistry.

2.2 Experimental setup

All experiments were conducted in culture vessels consisted of water-jacketed 2 l cylinders (filled to 1.8 l) connected to a circulating water bath maintained at a constant temperature of $20 \pm 0.6\,°\text{C}$ (light conditions as described in 2.1). Before experimental utilisation, the culture vessels were cleaned and filled completely with a 10 % HCl solution. After incubation for 24 h the HCl was removed and the vessels were rinsed with MilliQ-water and subsequently with sterile seawater (adjusted to the target carbonate system). Carbon dioxide concentrations were achieved by mixing CO_2 free air with pure carbon dioxide (Air Liquide, France) using mass flow controllers (ANALYT-MTC Model 35823 and

Brooks Model 5850 TR) and an air pump (flow rate of $100 \pm 10\,ml\,min^{-1}$). CO_2 free air was generated by pumping ambient air through an activated carbon filter device to remove organics (Whatmann Carbon Cap) and subsequently passed through soda lime to remove carbon dioxide. This procedure was efficient enough to produce an air stream containing less than 2 ppm CO_2. The "CO_2-free" air was periodically checked using a Licor CO_2 analyser (LI-820) calibrated with a 400 ppm CO_2-air mixture (Deuste Steiniger, Germany). Precision of the Licor CO_2 analyser was 1.5 %. Carbon dioxide concentration in the CO_2-air stream was monitored every second with a Licor CO_2 analyser. The experimental setup created an oscillation around the target CO_2-value of ≈ 14 % (RSD, $n = 12$).

2.2.1 Batch experiments

Batch experiments were performed in triplicate for each pCO_2 treatment (B1, B2 and B3). 1800 ml of culture medium (see above but excluding the nutrient additions) was filled into each culture vessel through a 0.2 µm sterile and acid cleaned filter, leaving an atmosphere of 200 ml. The culture medium was bubbled with the target pCO_2 stream for 4 days (pCO_2 of ≈ 280, ≈ 400, ≈ 1000 µatm). Afterwards, aeration was relocated to the atmosphere of the culture vessel keeping the target pCO_2 over the culture medium surface. The salinity of the culture medium was increased to 38.5 due to the aeration with dry CO_2 air and subsequent evaporation of ≈ 25 ml. Nutrients were added to the culture medium according to f/20 (Guillard, 1975) and an exponential growing preculture of *E. huxleyi* was inoculated to a cell density of $1000\,cells\,ml^{-1}$. After gently mixing by a magnetic stirrer, samples were taken for dissolved inorganic carbon (DIC), total alkalinity (TA) and bacterial abundance. The exponential growing population of *Emiliania huxleyi* was allowed to grow for 5 to 6 generations (final cell density $< 7 \times 10^4\,cells\,ml^{-1}$) under the experimental conditions (≈ 4 days) and subsequently the incubation was terminated for sampling. Samples were taken for DIC, TA, cell number, coccosphere/cell diameter and coccolith volume, particulate organic phosphate (POP), particulate organic carbon (POC), total particulate carbon (TPC), bacterial abundance and scanning electron microscopy (SEM). Cell numbers were measured before and after sampling to account for the increase in cell number during the 2 h procedure.

2.2.2 Chemostat experiments

Culture media for the chemostat experiments (C1, C2 and C3) were prepared in 20 l polycarbonate tanks (chemostat supply tanks), which were prewashed with HCl and autoclaved before utilisation. Seawater was aerated with the target pCO_2 -air stream (as described above) for 1 week to assure equilibrium of the carbonate system. Afterwards, sterile filtered nutrients were added to the chemostat supply

tanks according to f/20, except for the nitrate concentration which was set to 9.0 ± 1.4 µmol l^{-1} (1SD, $n = 3$) resulting in a N:P ratio of ≈ 2.5. Culture medium was transferred from the chemostat supply tanks to the precleaned culture vessels (chemostats) via acid cleaned tubes passing a 0.2 µm sterile acid cleaned filter. Prior to medium transfer, the chemostats were cleaned with HCl, subsequently rinsed with deionised water and culture medium (see Sect. 2.2). When the chemostats were filled with 1800 ml of culture medium, the supply was stopped and an exponential growing preculture of *E. huxleyi* was inoculated. *Emiliania huxleyi* ($1000\,cells\,ml^{-1}$) was allowed to grow exponentially to the maximum population density (limited by the nitrate concentration of 9.0 ± 1.4 µmol l^{-1}) and afterwards the medium inflow (dilution rate) from the supply tanks to the chemostats was restarted. The chemostats were operated at a constant dilution rate ($D = 0.49 \pm 0.01\,d^{-1}$), which was periodically checked by weighting the incoming medium. After 7 to 10 days under nitrogen limitation (acclimation period), *E. huxleyi* reached equilibrium state conditions (constant cell number) and was allowed to grow for another 10 days under equilibrium before the dilution was stopped and the chemostat culture was sampled. Cell number, coccosphere/cell diameter and coccolith volume were checked daily with a Coulter Multisizer™3. Samples were taken for DIC, TA, cell number, coccosphere/cell diameter and coccolith volume, POP, POC, TPC, nutrient concentration (nitrate + nitrite and phosphate), bacterial abundance and SEM. Additionally, DIC and TA were sampled from the media supply tank every second day during the equilibrium state (total sample number of five).

2.3 Carbonate system analysis

The carbonate system was monitored by TA and DIC measurements. DIC samples (25 ml) were taken carefully in duplicate with a disposal single use syringe, avoiding air contact, and filtered through a sterile filter (pore size 0.2 µm). Samples were sealed air tight, stored at 4°C in the dark and measured within one month after sampling. Duplicate DIC samples were analysed as the mean of triplicate measurements with the infrared detection method by using AIRICA (MARIANDA, Germany) and corrected to Dickson seawater standards. Consecutive measurements of the Dickson standard resulted in an average precision of ± 0.08 % (1RSD, $n = 15$).

Samples for TA (100 ml) were sterile filtered (0.2 µm pore size) and stored dark at 4°C prior to analysis (within 5 days after sampling). TA was measured in duplicate by the potentiometric titration method after Dickson (1981) and corrected to Dickson seawater standards. Consecutive measurements of the Dickson standard resulted in an average precision of ± 3.6 µmol kg^{-1} (1SD, $n = 14$). The carbonate system was calculated by using the program CO2sys (version 1.05 by
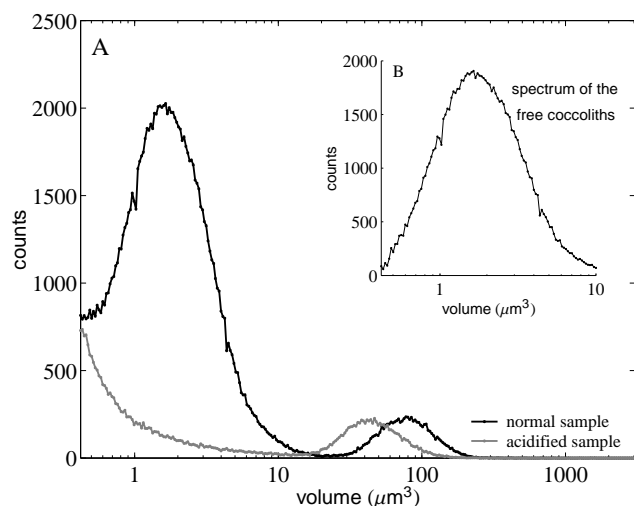
Fig. 1. Multisizer™ 3 volume spectra. **(A)** Spectra of the *E. huxleyi* population (black line) and after treatment with HCl (grey line). **(B)** Volume spectrum of the free coccoliths after subtracting the acidified sample from the normal sample.

E. Lewis and D. W. R. Wallace) with dissociation constants for carbonic acid after Roy et al. (1993).

2.4 Cell number, coccosphere/cell diameter and coccolith volume

Samples for cell number and coccosphere/cell diameter were processed directly after sampling and each measured three times with a Coulter Multisizer™3. Afterwards, the samples were acidified with $0.1\,\mathrm{mmol\,l^{-1}}$ HCl to dissolve all free and attached coccoliths and subsequently measured again to determine the cell diameter of *E. huxleyi* (Fig. 1a, grey line). Subtracting the acidified-sample-spectrum from the non-acidified-sample spectrum resulted in a spectrum to determine the average volume of the free coccoliths assuming normal distribution (Fig. 1b).

The mean cell number was used to calculate the growth rate μ ($\mathrm{d^{-1}}$) during the batch culture experiments as

$$\mu = \frac{(\ln c_1 - \ln c_0)}{t_1 - t_0} \tag{1}$$

where c_0 and c_1 are the cell concentrations at the beginning (t_0) and end of the incubation period (t_1), expressed in days. The growth rate in the chemostat experiments equals the dilution rate (D) under equilibrium conditions and therefore $\mu = D$.

The "Coulter Counter Principle" is based on changes in the resistance across a sensing zone. Changes in the resistance between the particle and the electrolyte are recorded as voltage or current pulses. The number of pulses can be transferred to the number of particles measured, whereas the amplitude is transferred to the volume of the particle. The measured volume can be translated to the particle's diame-

ter if the particle is of spherical appearance. This accounts for the cell and the coccosphere of *E. huxleyi* but does not apply for the coccoliths. In this regard, coccolith measurements are expressed in volume (μm^3) and coccosphere/cell measurements are expressed in diameter (μm).

Coccoliths of *E. huxleyi* morphotype A have a complex geometrical structure with a distal and proximal elliptical shield or plate connected by a central tube. The distal shield has a grid like structure with small "gaps" between the coccolith elements, whereas the proximal shield is solid. The Coulter Multisizer™3 is able to recognise these gaps if they are soaked and filled with the electrolyte (in this case: sterile filtered seawater).

The Multisizer™3 was calibrated with $5.1\,\mu m$ latex beads (Coulter®CC L5) and resulted in a precision of $\pm 0.03\,\mu m$ (1SD, $n = 20$).

2.5 Production rates of particulate inorganic and organic carbon, particulate organic phosphate and total particulate nitrogen

For each experiment, 4 sub-samples were filtered onto precombusted GF/F filters ($450\,°C$ for 7 h) and frozen at $-20\,°C$. TPC and POC were measured on separate filters using an "Euro EA Elemental Analyser" (Ehrhardt and Koeve, 1999). The filter for POC analysis was treated with fuming HCl ($\approx 10\,h$) to remove all inorganic carbon. Particulate inorganic carbon (PIC) was calculated from the difference of TPC and POC. Total particulate nitrogen (TPN) was analysed simultaneously with the TPC measurements. POP was measured by wet oxidation in acid persulfate (Koroleff, 1999). Cell quota of particulate matter PM (PM $\triangleq$ PIC, POC, POP and TPN) was calculated as

$$\frac{\mathrm{PM}}{\mathrm{cell}} = \frac{\mathrm{PM_{filter}}}{V \cdot N} \tag{2}$$

where $\mathrm{PM_{filter}}$ is the mass (pg) of particulate matter per filter, V is the volume (ml) filtrated and N is the number of cells per ml. Cell quota estimates are associated with an error of $< 5\%$. Production rates of PIC, POC, POP and TPN were calculated by multiplying the cell quota with the growth rate (μ). The TPC/TPN filter from chemostat experiment C1 was lost during the preparation and measurement procedure.

2.6 Nutrient measurements

Nutrients were sampled during the chemostat experiments and the sum concentrations of nitrite and nitrate were determined from the chemostat supply tanks and from the chemostats under equilibrium growth conditions. Additionally, phosphate concentrations were determined from the chemostats under equilibrium growth conditions to assure sufficient supply of phosphate. Samples were sterile filtered and stored at $-20\,°C$ until analyses. The concentration of nitrate/nitrite was measured with a precision of $\pm 0.1\,\mu mol\,l^{-1}$

using a Technicon Auto-analyser (Malara and Sciandra, 1991). Phosphate analyses were performed photometrically (precision of $\pm 0.03\,\mu mol\,l^{-1}$) according to Hansen and Koroleff (1999).

2.7 Bacterial abundance

Water samples for bacterial abundance were taken at the start and end of the batch experiments and at equilibrium growth condition of the chemostat experiments to estimate particulate organic carbon production by bacteria. Water samples were preserved with 2 % (wt/vol) formaldehyde and stained with 4'6-diamino-2-phenylindole (DAPI, final concentration $0.25\,\mu g\,ml^{-1}$) and filtered onto black $0.2\,\mu m$ polycarbonate filters (Porter and Feig, 1980). Total bacterial abundance was determined by direct counts. Between 500 and 600 bacteria were counted with an Axiophot-Zeiss epifluorescence microscope at $\times 1000$ magnification. Organic carbon due to bacterial biomass was calculated from bacterial abundance under the assumption of a carbon content of $30\,fg\,cell^{-1}$ (average for coastal samples according to Fukuda et al., 1998).

2.8 Scanning electron microscopy

Samples for SEM were filtered onto cellulose acetate filter ($0.45\,\mu m$ pore size) and afterwards dried at $60\,°C$ pending analyses. Sputter coated (Gold-Palladium) filter portions were observed on a Hitachi S-3000N SEM.

3 Results

Manipulation of the seawater carbonate system by changing DIC concentrations and keeping TA constant resulted in a pCO_2 range from 280 to $1080\,\mu atm$ and 210 to $1180\,\mu atm$ in the batch and chemostat experiments, respectively (Table 1). DIC consumption by biological activity in the batch and chemostat experiments was less than 5 % and 4 %, respectively.

A sufficient macro- and micronutrient concentration at the end of the batch experiments (nutrient replete) was assured by terminating the experiments at cell densities below $7 \times 10^4\,cells\,ml^{-1}$ and therefore keeping depletion of nutrients at a minimum (Table 2). During equilibrium conditions of the chemostat experiments $NO_3^- + NO_2^-$ concentrations were near or below the detection limit ($< 0.2\,\mu mol\,l^{-1}$), whereas PO_4^{3-} concentrations were above $1\,\mu mol\,l^{-1}$ (Table 2).

In comparison to replete nutrient conditions (B1–B3), production rates of POC and TPN were decreased by $\approx 50\%$ under nitrogen limitation (C1–C3) as a consequence of the reduced growth rate (μ) and lower cell quota (Tables 2 and 3). On the other hand, cellular POP quota increased under nitrogen limitation, whereas the POP_{prod} decreased (Tables 2 and 3). Bacterial POC contributed less than 2 % to the total POC during all experiments and can be therefore neglected.

Cellular growth rate under nutrient replete conditions was highest at intermediate pCO_2 and decreased towards higher and lower pCO_2 conditions (Table 3). Under nitrogen limitation, however, the growth rate was determined by the dilution rate of the chemostat resulting in no change over the applied pCO_2 conditions (Table 3).

In regard to increasing pCO_2, production rates and cell quota of POC, TPC and POP displayed a positive trend under replete nutrients and nitrogen limitation (Tables 2 and 3). PIC_{prod} was highest at nutrient replete and intermediate pCO_2 condition ($440\,\mu atm$) and decreased towards elevated pCO_2 ($1080\,\mu atm$) from 21.3 ± 3.2 to $12.1 \pm 2.9\,pgC\,cell^{-1}\,d^{-1}$. Under nitrogen limitation a lower PIC_{prod} was observed when elevating the pCO_2 from 544 to $1180\,\mu atm$ (4.0 to $3.7\,pgC\,cell^{-1}\,d^{-1}$). This decreasing trend was confirmed by applying the alkalinity anomaly technique (Sciandra et al., 2003), which resulted in an estimated calcification rate of 2.5, 2.4 and $2.2\,pgC\,cell^{-1}\,d^{-1}$ from low, over intermediate, to elevated pCO_2, respectively. PIC:POC ratio decreased with increasing pCO_2 levels in the batch and chemostat experiments (Table 3). POC:POP and TPN:POP ratios displayed an increasing trend with pCO_2 in all experiments, whereas the POC:TPN ratio did not significantly change under the applied pCO_2 levels (Table 3).

Both under nutrient replete and nitrogen limited conditions, coccosphere and cell diameter increased with pCO_2 (Fig. 2) ranging from 3.98 ± 0.03 to $5.72 \pm 0.11\,\mu m$ and from 3.68 ± 0.03 to $4.92 \pm 0.21\,\mu m$, respectively (Table 4). Coccolith volume was substantially reduced under nitrogen limitation compared to nutrient replete conditions (Fig. 3, Table 4) and increased significantly from low to high pCO_2 under nutrient replete conditions. Repeated daily measurements ($n = 10$) of coccolith volume during equilibrium conditions (nitrogen limitation) revealed an increasing trend from low/intermediate to high pCO_2 (Table 4).

4 Discussion

The batch experiments (nutrient replete) were designed to provide optimal growth conditions (excepting the variations in the carbonate system). This was achieved by providing saturated light conditions, an optimal temperature for *E. huxleyi* isolated from temperate regions (Paasche, 2002; Buitenhuis et al., 2008) and excess nutrients (see Sect. 2.2.1). Cellular growth in batch experiments is a very dynamic system. Biomass and cell number rapidly increase (exponential) changing the chemical composition of the growth medium over the course of the experiment (e.g. nutrient concentrations, carbonate system, irradiance due to self-shading etc.). However, chemical changes in the medium due to biological activity were kept to a minimum by terminating the batch experiments at low cell densities (Tables 1 and 2).

In the chemostat experiments, cellular growth was moderately limited by the supply of nitrogen in form of nitrate

Table 1. Carbonate system parameters from the batch and chemostat experiments. Values from the batch cultures are expressed as mean values with according standard deviation calculated from start and end measurements of the experiments (1SD, $n = 6$). Values from the reservoir tanks (chemostat experiments) are expressed as mean with according standard deviation under equilibrium conditions (1SD, $n = 5$).

exp. code	DIC (μmol kg^{-1})	TA	$p\text{CO}_2$ (μatm)	pH (total scale)	Ω (calcite)	CO_2	HCO_3^- (μmol kg^{-1})	CO_3^{2-}
			Batch experiments					
B1	2098 ± 30	2514 ± 19	282 ± 21	8.20 ± 0.02	6.9 ± 0.2	9 ± 0.6	1792 ± 38	297 ± 10
B2	2214 ± 109	2525 ± 123	442 ± 15	8.04 ± 0.01	5.3 ± 0.3	14 ± 0.5	1971 ± 96	228 ± 13
B3	2314 ± 56	2447 ± 86	1077 ± 165	7.70 ± 0.08	2.7 ± 0.5	34 ± 5.2	2163 ± 43	117 ± 23
			Chemostat experiments					
supply tanks								
C1	2068 ± 10	2577 ± 4	207 ± 6	8.31 ± 0.01	8.4 ± 0.1	7 ± 0.2	1701 ± 14	361 ± 4
C2	2260 ± 23	2580 ± 2	445 ± 48	8.05 ± 0.04	5.5 ± 0.4	14 ± 1.6	2010 ± 38	236 ± 17
C3	2431 ± 3	2582 ± 3	1022 ± 33	7.74 ± 0.01	3.0 ± 0.1	32 ± 1.0	2268 ± 5	130 ± 4
chemostats								
C1	1988	2428	235	8.25	7.2	7	1673	308
C2	2189	2441	544	7.96	4.4	17	1984	187
C3	2368	2485	1180	7.67	2.5	37	2222	108

Table 2. Overview of cell density, cell quota of particulate nitrogen/phosphorus and culture media nutrient concentrations ($NO_3^- + NO_2^-$ and PO_4^{3-}) at the end of the batch and chemostat experiments. $NO_3^- + NO_2^-$ and PO_4^{3-} concentrations at the start of the batch experiments (B1–B3) were $\approx 88.2 \, \mu$mol l^{-1} and $\approx 3.6 \, \mu$mol l^{-1}, respectively. $NO_3^- + NO_2^-$ and PO_4^{3-} concentrations of the reservoir tanks during the chemostat experiments (C1-C3) were $9.0 \pm 1.4 \, \mu$mol l^{-1} and $\approx 3.6 \, \mu$mol l^{-1}, respectively.

exp. code	$p\text{CO}_2$ (μatm)	cell no. (cells ml^{-1}) $\times 10^4$	TPN (pgN cell^{-1})	POP (pgP cell^{-1})	$NO_3^- + NO_2^-$	PO_4^{3-} (μmol l^{-1})
			Batch experiments			
B1	282 ± 21	5.1 ± 1.5	1.34 ± 0.21	0.20 ± 0.03	$*83.4 \pm 0.7$	$*3.28 \pm 0.04$
B2	442 ± 15	5.4 ± 3.2	2.00 ± 0.60	0.20 ± 0.02	$*81.4 \pm 1.5$	$*3.26 \pm 0.18$
B3	1077 ± 165	6.1 ± 2.6	3.40 ± 0.53	0.31 ± 0.06	$*73.9 \pm 4.7$	$*3.01 \pm 0.21$
			Chemostat experiments			
C1	235	6.8 ± 0.6	–	0.33	0.1	1.08
C2	544	6.1 ± 0.6	1.10	0.35	0.1	1.07
C3	1180	5.8 ± 0.4	1.64	0.38	0.2	1.78

* Values were estimated by subtracting the TPN and POP bulk production (determined from cell densities and cell quota) from the nutrient concentration at the beginning of the batch experiments.

(dilution rate of 0.49 ± 0.01 d^{-1}) and equilibrium growth conditions (constant cell number/biomass) were achieved over several generations. The concentration of nitrogen (nitrate and nitrite) during equilibrium state was below or near the detection limit which stringently does not prove nitrogen limitation. In chemostat theory, the concentration of the limiting nutrient rises when the supply rate (dilution rate) exceeds the maximum uptake rate of the organism (maximum growth rate). The applied dilution rate of 0.49 ± 0.01 d^{-1} was far lower than maximum growth rates of *E. huxleyi* under similar conditions (Buitenhuis et al., 2008). This and the low cellular TPN quota (as discussed below) led to the conclusion that nitrogen was the limiting factor during the conducted chemostat experiments. The maximum biomass and cell number at equilibrium conditions were kept low in comparison to common conducted experiments where a high biomass is desired. Thus, changes in the carbonate chemistry due to biological activity were relatively small (see Table 1, comparing the supply tanks with the chemostats).

Table 3. Physiological parameters and cellular ratios of the batch (1SD, $n = 3$) and chemostat experiments. Significance was tested for the batch culture experiments using a one-way ANOVA ($p < 0.05$).
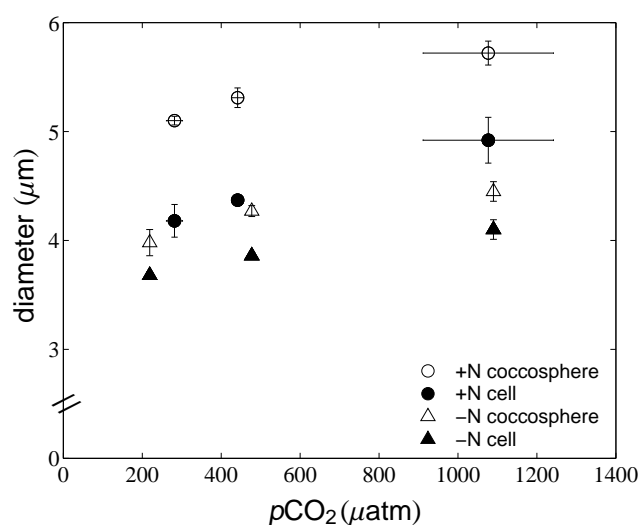
exp. code	μ (d^{-1})	PIC$_{prod}$ (pgC cell^{-1}d^{-1})	POC$_{prod}$	TPN$_{prod}$ (pgN cell^{-1}d^{-1})	POP$_{prod}$ (pgP cell^{-1}d^{-1})	PIC:POC	POC:TPN (mol : mol)	POC:POP	TPN:POP
				Batch experiments					
B1	1.02 ± 0.07	15.7 ± 1.8	11.3 ± 1.0	1.36 ± 0.12	0.20 ± 0.02	1.41 ± 0.27	9.68 ± 0.70	142 ± 5	14.8 ± 0.9
B2	1.32 ± 0.09	21.3 ± 3.2	16.3 ± 2.6	2.67 ± 0.93	0.27 ± 0.04	1.31 ± 0.11	7.55 ± 1.83	159 ± 11	21.9 ± 5.7
B3	0.94 ± 0.12	12.1 ± 2.9	25.6 ± 2.8	3.16 ± 0.11	0.29 ± 0.02	0.47 ± 0.08	9.45 ± 1.13	231 ± 41	24.4 ± 1.6
p	< 0.01	0.02	< 0.001	0.02	0.02	< 0.01	0.17	< 0.01	0.03
F	12.86	8.87	29.99	8.81	7.92	25.78	2.41	10.98	6.29
				Chemostat experiments					
C1	0.49 ± 0.01	–	4.5	–	0.16	–	–	73.4	–
C2	0.49 ± 0.01	4.0	5.4	0.54	0.17	0.75	11.7	79.6	6.8
C3	0.50 ± 0.01	3.7	7.6	0.82	0.19	0.48	10.8	104	9.6

Table 4. Coccosphere/cell diameter and coccolith volume from the batch and chemostat experiments. Significance was tested for the batch culture experiments (1SD, $n = 3$) using a one-way ANOVA ($p < 0.05$). Standard deviation for the chemostat experiments were derived from 10 repeated measurements during the equilibrium state (one sampling per day over the course of 10 days).

exp. code	pCO$_2$ (µatm)	coccosphere diameter (µm)	cell (µm)	coccolith volume (µm^3)
		Batch experiments		
B1	282 ± 21	5.10 ± 0.04	4.18 ± 0.15	1.50 ± 0.14
B2	442 ± 15	5.31 ± 0.09	4.37 ± 0.05	2.06 ± 0.39
B3	1077 ± 165	5.72 ± 0.11	4.92 ± 0.21	2.89 ± 0.56
p		< 0.001	< 0.01	0.015
F		39.4	19.0	9.1
		Chemostat experiments		
C1	235	3.98 ± 0.03	3.68 ± 0.03	0.51 ± 0.07
C2	544	4.27 ± 0.05	3.86 ± 0.03	0.50 ± 0.01
C3	1180	4.45 ± 0.09	4.10 ± 0.09	0.63 ± 0.03

Fig. 2. Coccosphere (open symbols) and cell (filled symbols) diameter under nutrient replete (+N, circles) and nitrogen limitation (−N, triangles) in regard to pCO$_2$ levels.

4.1 Cellular rates and ratios

A reduction in cellular TPN quota of about 50 % was measured during chemostat equilibrium conditions compared to batch culture conditions indicating cellular nitrogen limitation induced by the applied low medium $NO_3^- + NO_2^-$ inflow (Table 2). Additionally, cellular ratios (POC:TPN, POC:POP and TPN:POP) were similar to previously reported values from chemostat studies using nitrogen limitation with low N:P medium inflow (Leonardos and Geider, 2005). Interestingly, TPN:POP and POC:POP ratios decreased under nitrogen limited compared to nutrient replete conditions while

POC:TPN ratios remained unchanged (Table 3) which suggests that the phosphorus metabolism was partly decoupled from the C and N dynamics.

The response in photosynthesis of *E. huxleyi* to changing carbonate chemistry has been studied intensively in laboratory experiments over the last decades and summaries are given in Riebesell and Tortell (2011), Hoppe et al. (2011) and Ridgwell et al. (2009). Most laboratory experiments were performed under nutrient replete conditions (Riebesell et al., 2000; Langer et al., 2009; Feng et al., 2009; Hoppe et al., 2011). In the tested pCO$_2$ range from 282 to 1077 µatm, POC$_{prod}$ increased similar to previous findings under replete nutrient conditions (Riebesell et al., 2000; Zondervan et al., 2002; Barcelos e Ramos et al., 2010). A few laboratory
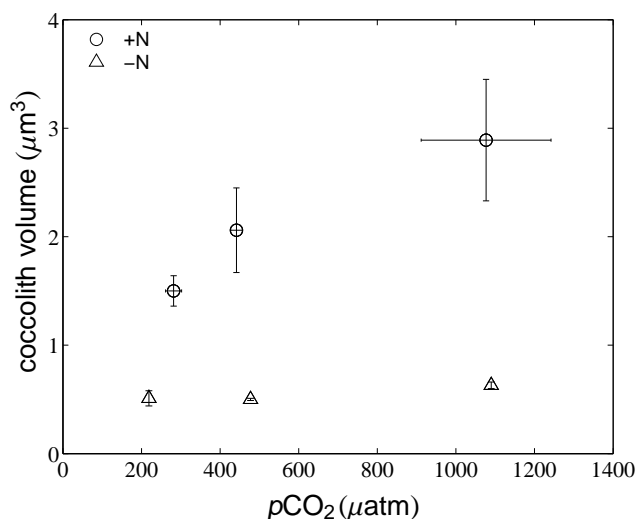
Fig. 3. Coccolith volumes under nutrient replete (+N, circles) and nitrogen limitation (−N, triangles) in regard to the applied pCO_2 levels.

studies dealt with the combined effect of rising pCO_2 and nitrogen limitation (Sciandra et al., 2003; Leonardos and Geider, 2005). In contrast to Sciandra et al. (2003) a positive trend in POC_{prod} was observed as reported by Leonardos and Geider (2005) for a non-calcifying strain of *E. huxleyi*. Differences in the experimental set up of Sciandra et al. (2003) and this study might be an explanation for the diverging response observed in POC_{prod}. In the current study, the biomass during chemostat equilibrium condition was of about 10 times lower and the growing culture was not bubbled with a gas mixture which might interfere with phytoplankton growth and performance (Shi et al., 2009). Additionally, high light intensities (as used here and in Leonardos and Geider, 2005) are known to amplify the positive effect of pCO_2 on POC_{prod} (Zondervan et al., 2002). Increasing POC_{prod} was accompanied by increasing trends in TPN_{prod} and POP_{prod} (Table 3), whereas the TPN:POC ratio displayed no change with pCO_2 (Müller et al., 2010). Cellular ratios of POC:POP and TPN:POP increased with pCO_2 and led to a change of the canonical stoichiometry (C:N:P) of *E. huxleyi* (Table 3). The extent of change in the canonical stoichiometry depends primarily on the C:N:P supply and the cellular growth rate (Klausmeier et al., 2008). However, experimental evidence from mesocosm and laboratory experiments indicate the importance of pCO_2 for the stoichiometry of *E. huxleyi* under projected ocean acidification/carbonation (Leonardos and Geider, 2005; Engel et al., 2005; Schulz et al., 2008).

The response in PIC_{prod} and PIC:POC of *E. huxleyi* was negatively affected by elevated pCO_2 under nutrient replete conditions (Table 3). These results are in line with the commonly described strain specific sensitivity of *E. huxleyi* to pCO_2 (Bach et al., 2011; Findlay et al., 2011; Barcelos e Ramos et al., 2010; Langer et al., 2009).

4.2 Coccosphere/cell diameter and coccolith volume

Coccosphere and cell diameter were reduced by $> 0.5\,\mu m$ ($>10\,\%$) under nitrogen limited compared to nutrient replete conditions (Table 4). Similar effects of nitrogen depletion on coccosphere/cell diameter of *E. huxleyi* were previously observed and reported in the literature (Paasche, 2002; Sciandra et al., 2003) and reflect substrate limitation and reduced accumulation of biomass.

In regard to pCO_2, coccosphere/cell diameter increased by about $0.08\,\mu m$ and $0.05\,\mu m$ per $100\,\mu atm$ pCO_2 under nutrient replete and nitrogen limited conditions, respectively (assuming a linear correlation). However, the observed increase in coccosphere/cell diameter with pCO_2 can only be applied within the tested range as recent results indicate a steady decrease in coccosphere diameter at pCO_2 values above $> 1500\,\mu atm$ (Bach et al., 2011).

In contrast to our study, De Bodt et al. (2010) (nutrient replete conditions) and Sciandra et al. (2003) (nitrogen limited conditions) reported a decrease in coccosphere diameter of *E. huxleyi* within a comparable pCO_2 range. These diverging observations are presumably an effect of the unaltered (De Bodt et al., 2010) and decreased (Sciandra et al., 2003) POC_{prod} which in combination with a diminished PIC_{prod} might lead to a reduction in coccosphere diameter. Additionally, the latter experiments were performed under lower light intensities ($150–170\,\mu mol\,photons\,m^{-2}\,s^{-1}$) than compared to the present study ($300\,\mu mol\,photons\,m^{-2}\,s^{-1}$). Increasing light intensities are reported to amplify the positive effect of pCO_2 on the POC_{prod} of *E. huxleyi* (Zondervan et al., 2002). Another point might be the optical measuring principle (HIAC) to determine the coccosphere diameter used in Sciandra et al. (2003) compared to the resistive method (Beckman Coulter). The coccosphere highly scatters and reflects light of different wavelengths which might interfere with measurements based on attenuation and absorption of light. For example, a thinning of the coccosphere layer surrounding the cell might result in a lower light scatter and therefore in an optical-measured decrease in diameter even if the coccosphere diameter is unaltered due to an concomitant increase in the cell diameter.

Changes in the average cell diameter of *E. huxleyi* due to changing oceanic conditions (warming, acidification, stratification, nutrient supply etc.) will have direct implications on metabolic rates, nutrient diffusion/uptake, grazing, sinking rates and should be considered when predicting broader ecological processes (Engel et al., 2008; Finkel et al., 2010).

In the present study, measured coccolith volume (via Coulter Multisizer™3) ranged from 0.50 to $2.89\,\mu m^3$ which is comparable to volume estimates derived from coccolith length measurements via scanning electron microscopy (0.3 to $3.6\,\mu m^3$, Young and Ziveri, 2000). Converting the measured coccolith volume to coccolith mass using the density of pure calcite ($2.71\,pg\,\mu m^{-3}$) results in coccolith mass values from 1.4 to $1.7\,pg\,CaCO_3$ and 4.1 to $7.8\,pg\,CaCO_3$ per

coccolith under nitrogen limited (C1–C3, Table 4) and nutrient replete conditions (B1–B3, Table 4), respectively. Poulton et al. (2011) sampled coccoliths of *E. huxleyi* morphotype A in surface waters from the Patagonian Shelf and determined via SEM morphometrics an average coccolith mass of $1.9 \pm 0.7 \, \text{pg} \, CaCO_3$. This value is in good agreement with the present estimates derived from the nitrogen limitation experiments. On the other hand, calculated coccolith masses from the nutrient replete experiments (4.1 to $7.8 \, \text{pg} \, CaCO_3$) are higher than estimates from other laboratory studies (Paasche, 1998, 1999, 2002; Bach et al., 2012) which report values from 1.5 up to $4 \, \text{pg} \, CaCO_3$ per coccolith. Differences between the latter and the present study might be caused (1) by diverging experimental set up and the cultured strain/morphotype of *E. huxleyi* and/or (2) by the applied method to estimate coccolith mass (SEM, birefringence-based and resistive methods). The Coulter Multisizer™ method determines the actual volume of coccoliths and theoretically accounts for the gaps in the distal shield of the coccoliths. Organic particles and matter might be attached to the coccoliths and/or cover some of the gaps. In this case, the present volume measurements would be slightly overestimated. At the moment, none of the mentioned possibilities can be excluded but it is strongly recommended for future studies to provide geometric data of coccoliths (via SEM) in conjunction with volume (resistive, Coulter Multisizer™) and/or mass (birefringence-based (SYRACO), Beaufort, 2005) measurements. Unfortunately, inappropriate filter material and storage problems hindered an adequate coccolith analysis via SEM/SYRACO of the conducted experiments (see discussion below).

A significant positive increase in coccolith volume was detected with elevated $p\text{CO}_2$ under nutrient replete conditions and a similar trend was observed from low/intermediate to high $p\text{CO}_2$ under nitrogen limitation (Fig. 3, Table 4). The highest coccolith volume (and presumably coccolith mass) was found at high $p\text{CO}_2$ (low pH) and nutrient replete conditions. A similar phenomenon was observed in nutrient rich and low pH Chilean upwelling waters by Beaufort et al. (2011), who measured coccolith masses of *E. huxleyi* morphotype R with values $> 8 \, \text{pg} \, CaCO_3$. High coccolith volume/mass at elevated $p\text{CO}_2$ and low pH seems to be counterintuitive considering the predominantly reported decrease in the calcification rate of coccolithophores (Riebesell and Tortell, 2011; Hoppe et al., 2011; Langer et al., 2009; Ridgwell et al., 2009). However, coccolith mass or volume (one point in time) are not comparable to the rate of calcification or PIC_{prod} (change over time). A comparable phenomenon has been reported for phosphate limited cells of *E. huxleyi* which produce coccoliths with a higher calcite content than under nutrient replete conditions (Paasche, 2002). Even though the calcite content per coccolith, the PIC cell quota and the cell volume increases with phosphate limitation, the calcification rate or PIC_{prod} decreases (Riegmann et al., 2000; Müller et al., 2008).
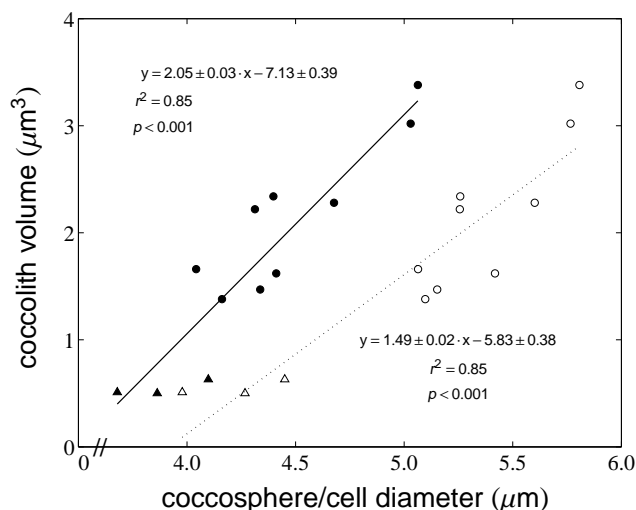

Fig. 4. Coccolith volume as a function of the cell (filled symbols) and coccosphere (open symbols) diameter. Circles and triangles indicate the batch and chemostat experiments, respectively.

Interestingly, estimated numbers of free/detached coccoliths during our experiments suggest a lower production of coccoliths per cell with elevated $p\text{CO}_2$, which would be an explanation for the decrease in PIC_{prod} with a concomitant increase in coccolith volume. However, only the free/detached coccoliths were measured and not the total produced coccoliths (free/detached + attached). Although samples were equally treated before analyses, a bias due to the physical treatment (rough or gentle mixing) or a change in coccolith density cannot be excluded.

In general, the coccolith volume was found to correlate with the coccosphere/cell diameter (Fig. 4). However, analysing the batch and chemostat experiments separately, the correlation is only applicable under nutrient replete conditions (batch experiments). This is presumably caused by the absence of additional data points to support such a relationship under nitrogen limited conditions. Therefore, the overall linear relationship between coccosphere/cell diameter and coccolith volume (in Fig. 4) should be interpreted as a first approximation. A power function relationship was recently indicated for the coccolith distal shield length (DSL) and the coccolith weight of *Coccolithus pelagicus* (Cubillos et al., 2012). If a similar power function exists connecting the coccosphere/cell diameter with the coccolith volume has to be validated in future studies. Experiments investigating the effect of various parameters (e.g. light, temperature and salinity) on different morphotypes and species will provide a suitable basis.

This study presents direct coccolith volume measurements from culture experiments and data for comparison are rare. However, coccolith distal shield length (DSL) has been described to correlate with the coccosphere diameter in field and fossil samples (Henderiks et al., 2012; Henderiks, 2008).
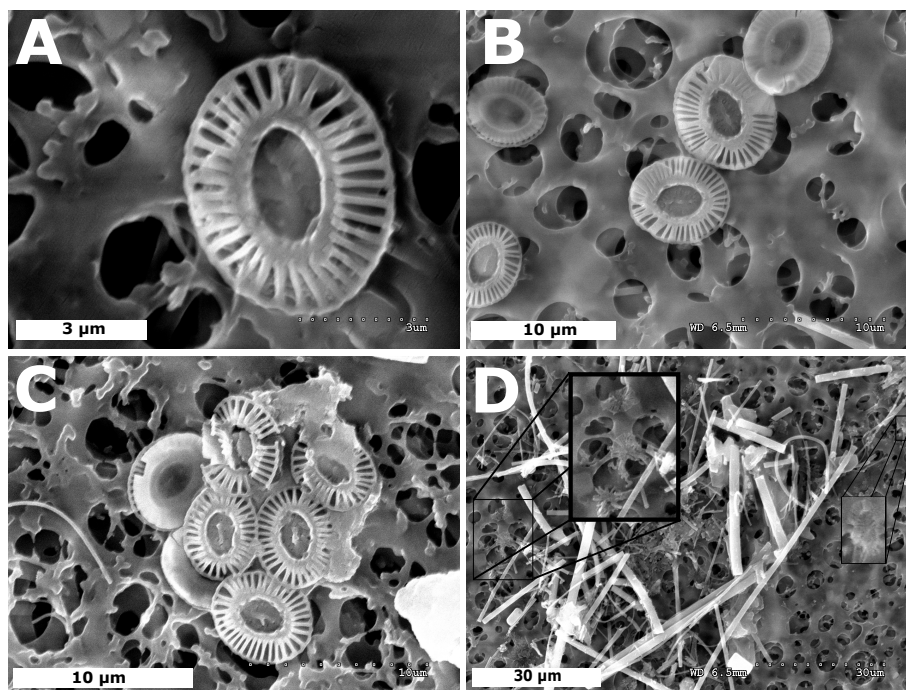
Fig. 5. SEM pictures of *E. huxleyi* cultures from the batch **(A–C)** and chemostat **(D)** experiments. Displayed are coccoliths produced by *E. huxleyi* exposed to a pCO$_2$ of **(A)** 282±21 µatm, **(B)** 442±15 µatm and **(C)** 1077±165 µatm. **(D)** Example from the chemostat experiments indicating disintegrated coccoliths (within the black rectangles) due to storage problems (see text for details).

Converting the measured coccolith volume (V) to distal shield length (DSL) by applying Eq. (2) with the species specific shape constant $k_s = 0.02$ (as given for normal calcified coccoliths of *E. huxleyi* morphotype A, Young and Ziveri, 2000) results in an average coccolith DSL ranging from 2.9 to 3.2 µm and 4.1 to 5.5 µm for the chemostat and batch experiments, respectively.

$$\mathrm{DSL}\,(\mu m) = \sqrt[3]{\frac{V\,(\mu m^3)}{k_s}} \qquad (3)$$

Corresponding to the estimates for coccolith mass the calculated DSLs from the batch experiments (nutrient replete) are higher than average DSL of ≈ 3.5 µm derived from field samples of *E. huxleyi* morphotype A (Henderiks et al., 2012; Poulton et al., 2011; Triantaphylloua et al., 2010; Young and Ziveri, 2000). Visual inspection of coccoliths from the batch culture experiments via scanning electron microscopy confirmed the presence of coccoliths with DSL > 4.1 µm (Fig. 5a–c), while coccoliths from the nitrogen limitation experiments (C1–C3) were found to be partly or completely disintegrated due to a preservation problem (Fig. 5d). Previous observations of *E. huxleyi* morphotype A with DSL > 4.1 µm (Cubillos et al., 2007) and the present SEM pictures let us assume that the calculated DSL (and consequently the coccolith volume) from the batch and chemostat experiments is valid and comparable to previous applied methods measuring the DSL of coccoliths.

A comparison of field and laboratory data on the relationship between coccosphere diameter and coccolith DSL of *E. huxleyi* (Fig. 6) reveals that results from laboratory experiments (Bach et al., 2012, and this study) have a distinct pattern from field data (Henderiks et al., 2012; Triantaphylloua et al., 2010). The difference between laboratory and field data is not surprising. Laboratory studies are commonly conducted with one single strain of *E. huxleyi* and environmental parameters are kept constant and optimised, except for one variable parameter (e.g. carbonate system or nutrient concentration). Field studies, on the other hand, are investigating whole *E. huxleyi* populations (assemblages of multiple strains) and several environmental parameters can change with time and space, amplifying or balancing their effect on physiology and coccolith formation. Additionally, environmental parameters can either influence directly physiology and coccolith formation or alter the strain distribution in one population towards a strain with a different coccolith geometry/morphology.

Figures 4 and 6 indicate the link between coccosphere/cell diameter and coccolith geometry (volume and DSL). It remains an open and interesting question if environmental parameters influence coccosphere/cell diameter and coccolith geometry separately or if coccolith geometry is indirectly influenced due to variations in coccosphere/cell diameter. Even an interaction of the two possibilities cannot be excluded.
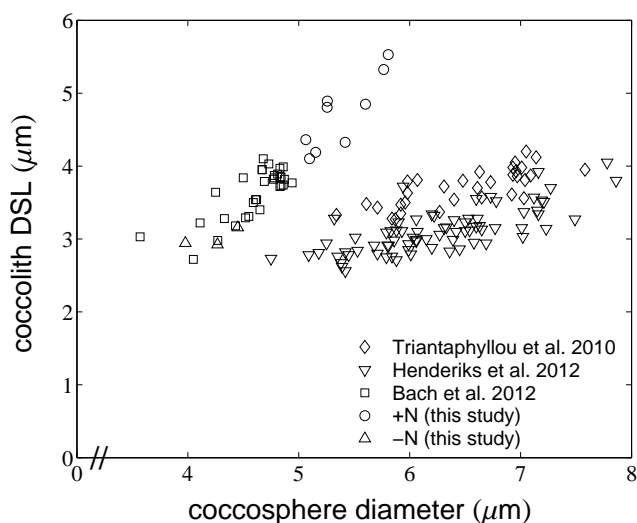
Fig. 6. *Emiliania huxleyi* coccolith distal shield length (DSL) as a function of coccosphere diameter from field and laboratory studies. Field data are from Mediterranean waters (*E. huxleyi* dominated samples, Triantaphylloua et al., 2010) and from the chlorophyll maximum near the cost of Namibia (Henderiks et al., 2012). Laboratory data are from a carbonate chemistry manipulation experiment (Bach et al., 2012) and this study. Details about measurement procedure and inherent errors can be found in the original studies.

Coccoliths of *E. huxleyi* are produced intracellular in the coccolith vesicle, a special cellular compartment, derived from the Golgi apparatus. After formation of the proto-coccolith ring, the coccolith matures inside the coccolith vesicle while transported to the cells surface. The time the coccolith matures inside the coccolith vesicle is presumably mainly influenced by the responsible metabolic rates and the distance to the cells surface. Therefore, an increase/decrease in cell diameter would provide additional/less time for coccolith growth and formation. Nitrogen limitation, for example, induces cell and coccolith shrinkage (Table 4, Paasche, 2002). Cell shrinkage increases the surface to volume ratio and hence the nutrient uptake efficiency but the cell has to maintain a certain cell diameter to pass on sufficient biomass and genetic material to the two daughter cells assuring their survival. To the contrary, phosphorus depletion inhibits DNA synthesis, while biomass buildup continuously results in an increase of coccosphere/cell diameter and coccolith size (Paasche, 2002; Müller et al., 2008). Indications are given that carbonate system parameters (e.g. pCO_2 and pH) have diverging effects on the coccosphere/cell diameter of *E. huxleyi*. Elevated pCO_2 conditions positively affect the cell diameter till a saturated level (reached at $\approx 1200\,\mu$atm), presumably due to an overconsumption of carbon (Bach et al., 2011; Engel et al., 2008). Increasing acidity (pH < 7.5 or $pCO_2 > 1500\,\mu$atm), on the contrary, negatively affects the cell diameter (Bach et al., 2011). The combination of both effects results in an optimum curve response of the cell di-

ameter to ocean acidification/carbonation (Bach et al., 2011, 2012).

It remains to be tested how changes in coccosphere/cell diameter induced by other environmental parameters (e.g. temperature, irradiance, salinity, trace metal availability) will influence coccolith volume or size. The complexity in coccolith size variability of natural observations as a result of various environmental parameters has been indicated (Herrmann et al., 2012; Poulton et al., 2011). A comparison of the different methods to estimate coccolith volumes and mass (birefringence-based, SEM and resistive method) is urgently needed to validate and confirm results on coccolith geometrics as previously mentioned by Poulton et al. (2011). The Coulter Multisizer™ method is an efficient and precise way to easily estimate the average coccolith volume from culture experiments by counting thousands of coccoliths within seconds. Controlled laboratory experiments will provide a suitable basis for method comparisons because sufficient sample material can be produced and experimental parameters are regulated and monitored.

This study was conducted with a desynchronised population of *E. huxleyi* induced by continuous light. It should be mentioned that measuring cell volumes under a light:dark cycle (synchronised division) has to be conducted with great care. Sampling has to be well timed because changes in cell diameter/volume occur within less than an hour.

5 Conclusions

It is demonstrated that the coccolith volume of *Emiliania huxleyi* varies with changes in the seawater carbonate chemistry but the effect is minor compared to a moderate nitrogen limitation. Coccolith volume was found to be primarily a function of the coccosphere/cell diameter. Indications are given that *E. huxleyi* produces more voluminous and lower number of coccoliths with increased pCO_2 resulting in a reduced particulate inorganic carbon production.

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